Reference: *Biol. Bull.*, **152**: 337–350. (June, 1977)

NUTRITIONAL REQUIREMENTS OF THE WATER FLEA *MOINA MACROCOPA*

DOUGLAS E. CONKLIN AND LUIGI PROVASOLI

Bodega Marine Laboratory, University of California, P.O. Box 247, Bodega Bay, California 94923; and Haskins Labs, Biology Department, Yale University, New Haven, Connecticut 06520

Herbivorous crustaceans comprise a significant proportion of filter-feeders; among them, Cladocera are found in almost all types of fresh waters. Although these abundant animals, known as water fleas, have been used extensively for a variety of research interests, little is known about their nutritional requirements.

Water fleas have been easily grown for a few generations on bacteria and algae, but maintaining high yields and continued fertility for many generations under agnotobiotic conditions is difficult. Rapid changes in microflora with attendant accumulation of metabolic products are often unfavorable and common in static cultures. A flowing system fed with algae grown in a chemostat offers better chances for stability and continued fertility (M. C. Glendening, University of Washington, personal communication).

The goal of sustained cultures can also be attained by growing Cladocera aseptically (D'Agostino and Provasoli, 1970; Murphy, 1970) on one or more algal species in media supplemented by organic nutrients and vitamin mixtures. Obviously, synxenic cultures, although successful, do not allow identification of nutritional factors for continued fertility.

Details on the composition and preparation of the artificial particulate media which support over 200 parthenogenetic generations of *Moina macrocopa americana* are given elsewhere (Conklin and Provasoli, in preparation). This study reports the nutritional requirements of *Moina* revealed during the substitution of crude nutrients by chemically defined constituents. Since the final medium has still one undefined component (liver infusion), definition of the minimal nutritional requirements has to be postponed.

Materials and Methods

Work on *Moina* was initiated after failure to grow *Daphina magna* on artificial media beyond one or two generations. Dr. James Murphy of the Rockefeller University suggested that *Moina macrocopa americana* might be more suitable, since it could be grown indefinitely on only one algal species as food organism; the other Crustacea tried, including *Daphnia,* required two algae (Murphy, 1970).

At first the inoculum was taken from maintenance cultures of *Moina* fed on *Chlamydomonas reinhardii* synxenically grown in a modified *Daphnia* medium (*i.e.*, the *mineral* part of the basal medium, Table I) or in DA medium (D'Agostino and Provasoli, 1970). Single young females served as inoculum after being freed of the accompanying algae by 7–10 serial transfers in 10 ml of mineral base, then fed overnight on the same medium with added particles of albumin-starch co-gel (SAgel, Table I) to cleanse their gut from algae.

TABLE I

Artificial media

Common balal medium (*per cent w or v/v*)

KCl, 3 mg; $MgSO_4 \cdot 7\ H_2O$, 4 mg; Ca (as Cl^-), 2 mg; K_3PO_4, 2 mg; $Na_2SiO_3 \cdot 9\ H_2O$, 2 mg; metal mix PII, 1 ml (1 ml contains Na_2EDTA, 1 mg; Fe, 0.01 mg; B, 0.2 mg; Mn, 0.04 mg; Zn, 0.005 mg; Co, 0.001 mg); Fe (as $(NH_4)_2$ H citrate), 0.05 mg; glycylglycine, 50 mg, pH 8.0 (TRIS buffer (Sigma Co.) is toxic for *Artemia*, *Daphnia* and *Moina* at 50 mg%. TES buffer (Sigma Co.) is nontoxic at 100 mg% for *Moina);* nucleic acid mix V, 2 ml (1 ml contains adenylic acid, 20 mg; guanylic acid, 10 mg; cytidylic acid, 10 mg; thymidine, 10 mg; dissolve in alkali, adjust to pH 8.0); DF 2, 1 ml (1 ml contains TWEEN 60, 2 mg; TWEEN 80, 2 mg; rutin, 0.5 mg; oxbile extract (Nutritional Biochem Co.), 1 mg.; disperse and emulsify components; adjust to pH 8.0); Cholesterol, 0.6 mg (dissolved in 95% ethanol, squirted into boiling water, ethanol boiled off; forms fine crystalline precipitate); amino acids mix III, 1 ml (1 ml contains L-isoleucine, 10 mg; L-lysine HCl, L-glutamic acid, L-histidine base, L-threonine, L-methionine, L-leucine, L-valine, L-proline, 1 mg each; L-arginine base, L-tyrosine, L-serine, glycine, L-tryptophane, 0.5 mg each); vitamin mix M1B, 1 ml (1 ml contains thiamine HCl, 0.5 mg; nicotinamide, 1.5 mg; pyridoxine HCl, 0.2 mg; biotin, 0.06 mg; putrescine·2 HCl, 0.1 mg; Vitamin B_{12}, 0.002 mg; choline H_2 citrate, 0.2 mg; riboflavin, 0.2 mg; folic acid, 0.1 mg; Ca pantothenate, 4 mg); Liver infusion L 25 (Oxoid, Flow Labs, Rockville, Md.), 70 mg (does not dissolve completely; upon autoclaving in medium forms a brown precipitate essential for growth). Adjust pH of basal medium to pH 7.6–7.8.

Particulate additions (*per cent*)

Essentially the particles are formed by dissolving or emulsifying the components in water; coagulating the mix by autoclaving; homogenizing the coagulum to produce fine particles (2–20 μm); autoclaving the particle slurry and homogenizing it again. Homogenization of particles containing lipids is done under flowing nitrogen; the mix is stored in well closed containers with N; add 1 drop preservative (O-fluorotoluene, 1 part; 1.2 dichloroethane, 1 part; 1 chlorobutane, 3 parts; Eastman Organic Chemicals) and store in refrigerator; active for 1–2 months. [Details for preparation of particles are given in Conklin and Provasoli, in preparation.]

E medium: Basal medium + trigel particles 2 ml [supplying egg albumin, 15 mg; rice starch, 10 mg; dry beef serum, 5 mg] + egg particles, 0.2 ml [supplying egg yolk, 10 mg; vitamin E (type II), 2 mg; calciferol, 0.5 mg].

FP medium: Basal medium + trigel particles 2 ml (as above) + FP particles 1 ml [supplying albumin fraction V, 4.5 mg; vitamin E (type II), 3 mg; egg lecithin, 1.5 mg; calciferol, 0.75 mg].

F1 medium: Basal medium + SA gel particles 2 ml [supplying egg albumin, 15 mg; rice starch, 10 mg] + FV particles 1 ml [supplying albumin fraction V, 6 mg; egg lecithin, 1.5 mg; BHT (butylated hydroxytoluene), 1 mg; calciferol, 1 mg; β-carotene, 0.5 mg; *dl*-α-tocopherol, 2 mg; linolenic acid, 1.5 mg; linoleic acid, 1 mg; palmitic acid, 1 mg; oleic acid, 0.5 mg].

This time-consuming operation was obviated when *Moina* was maintained on a medium containing egg-yolk particles and, later, on the more nearly defined artifical medium. In these media parthenogenetic females produced a brood of 4–6 newborn every other day.

For uniformity of inoculum, a few fertile females without visible eggs were transferred into tubes of artificial media; within the second day they produced a brood. The females were transferred to new tubes leaving behind their brood. Two days later these newborn became young females and were transferred singly to the experimental test tubes (10 ml, 25 × 120 mm). To insure rapid and uniform

growth, the tubes were shaken daily on a Vortex Genie (Scientific Industries, Inc.) to resuspend the fine particulates. The animals were not harmed by shaking; nevertheless, each tube was inspected to insure that no animal was trapped on the side of the test tube above the medium.

The test tubes were incubated at 25° C in very dim continuous light to avoid rapid destruction of light-sensitive vitamins (riboflavin and folic acid).

To reduce inherent variability of the inoculum (consisting of individuals born within 2 days, in a life-cycle lasting only 4–7 days), day 1 of each tube was the day in which the animal released the first brood. To score the effectiveness of each nutritional variable, the animals were killed on the seventh day by adding one drop of 1 ml % formalin and counted under the dissecting microscope in a plastic Bogarov counting tray (Wickstead, 1965).

Ten tubes of each experimental variable were inoculated with one juvenile; of these, seven tubes were counted on the seventh day and the count averaged; the remaining three tubes were killed and counted when the food particles had been almost completely exhausted (80–85% transmittance). The seven-day period allowed the first two broods of the inoculated female to reach maturity and to produce their first brood on day 7. The combined three generations growing in each test tube consisted of 13–19 adult females and 90–110 newborn and juveniles.

This procedure led, nonetheless, to variability in counts. Variations due to media preparation could be excluded, since all the variable consitutents of one experiment were added to portions of a common basal medium before being dispensed.

Some variability could be ascribed to the inoculum. Virgin females were not used to prepare inocula; hence, progeny could range from the first to fourth brood. Murphy and Davidoff (1972) have shown that early broods are less exacting in nutritional requirements, perhaps because of receiving more nutrients from the mother. However, considerable variability was also noted in this study among individuals in the same brood.

A "biological conditioning" occurred in nutritionally adequate media. Newborn, inoculated singly in a new test tube, generally took seven days from birth to become adult females and produce the first brood; these first brood individuals reached maturity and produced the first brood in only four days. The size of the brood also varied: the original female produced 4–6 newborn, which in turn produced a larger brood (6–8); the second and third brood of the original female might also be 6–8.

Choice of the seventh day for counting was arrived at empirically. Nutritionally complete media on day 7 gave counts ranging from 90–120 individuals, while counts from media lacking an essential nutrient were about 10–30; counts corresponded well to changes in concentration of essential nutrients. This indicated that the nutritional carry-over from the original juvenile female had been largely offset by the effect of the medium on her and her progenies' time to adulthood and brood size. Serial transfers would have provided a complete definition of the requirements if the medium was completely defined. Differences within a nutritional variable were reduced by averaging the counts of seven test tubes; however, if the range was large, the variable was retested. The remaining three replicate test tubes were allowed to go to exhaustion of the particles; such tubes served as extra controls to gauge whether the large variability in the seven counts was an artifact

due to the compounded effects of a delay of a few hours in the hatching of the F_1 and F_2 broods.

The artificial media were biphasic, and consisted of a liquid and a particulate phase (Table I).

Results

Initial attempts to grow a freshwater parthenogenetic cladoceran were as noted, with *Daphnia magna*. Profiting from experience, we added to the mineral base for *Daphnia* (D'Agostino and Provasoli, 1970) the components of the defined medium for *Artemia* (Provasoli and D'Agostino, 1969), *i.e.*, B vitamins, nucleic acids, cholesterol and starch-protein particles. Only occasional adults were obtained at first; after arriving at more suitable concentrations of the components, adults were regularly obtained but produced fertile eggs only sporadically. Incorporation of beef serum into the starch-protein particles (tri-gel, Table I) resulted in fertile adults but most nauplii never reached adulthood. Upon the advice of Dr. Murphy the trigel medium was tried on *Moina;* two fertile generations and, occasionally, a third generation were obtained.

The manifest improvement of fertility by serum addition focused attention on lipids. Coagulated egg-yolk particles improved growth and survival of newborn and the brood size of *Moina* and allowed an occasional second generation of *Daphnia*. Following the lead of Viehoever and Cohen (1938), vitamin E was incorporated in the egg yolk particles; six fertile generations of *Moina* ensued. Finally addition of vitamin D led to continuous growth of *Moina* (>200 generations). Since only six consecutive generations of *Daphnia* could be obtained in the best medium, *Daphnia* was set aside.

The egg medium (Table I) was used from then on for maintenance and for production of inocula and served as a yardstick in experiments leading to more defined media.

Since the vitamin E added to egg yolk (Type II of Sigma Chemical Co.) is a mixture of 50% vegetable oils and 50% dl-α-tocopherol, we sought to replace it with its components. But combinations with several vegetable oils and dl-α-tocopherol were inferior to "type-II" concentrate. Attempts to replace the egg yolk particles by incorporating vitamin E type II and vitamin D into the trigel failed but led to resolution of the impasse. The lipid fraction proved too large to be entrapped during coagulation by the albumin and serum proteins; it leached into the medium. Better retention of the lipids was sought by employing more albumin in the trigel, but still the oils separated. Finally the lipids were incorporated into a separate particle, exploiting the fat-binding properties of albumin fraction V. This new particle (FP, Table I) replaced egg yolk in maintaining viability of successive generations but resulted in smaller broods and longer time to adulthood.

The decision to incorporate all the lipid factors in one type of particle and to remove the serum from the tri-gel proved decisive. We regained ability to experiment with the starch : protein : lipid ratios, and to improve the lipid components and ratios within a separate particle. Eventually, with incorporation of lecithin and a suitable fatty-acid mixture, the new lipid-albumin particle (FV, Table I) replaced completely serum and egg yolk.

The nearly-defined medium (F1) has minerals, trace metals, amino acids, nucleic acids, B vitamins, and liver infusion in the liquid phase; an emulsion of TWEENS and bile salts (DF_2); a fine suspension of cholesterol crystals; and two larger particles (2–20 μm), the protein-starch co-gel (SA) and the complex FV particle containing egg lecithin, vitamin A, D, and E, four fatty acids and an antioxidant emulsified and entrapped in the heat-coagulated albumin fraction. The last undefined component of the medium is the Oxoid liver infusion which improves growth and fertility.

The results presented here derive from experiments aimed at replacing efficiently the unknowns that contributed to high, continuous reproduction. Experiments to define the minimal essential requirements were postponed because complete replacement of the liver infusion was not achieved. However, the data in the tables were obtained with the nearly defined F1 medium.

The mineral base seems quite adequate. Sporadic changes in the concentrations of the major elements and replacement with other trace metal mixtures effected no substantial improvement.

The vitamin requirements were analyzed using the egg particles and, later, the FV particles (Table II). The results were quite similar: thiamine, nicotinamide, pyridoxine, and pantothenic acid were clearly essential. Omission of folic acid and riboflavin permitted some growth and low fertiliy—as expected—for the medium had 70 mg% of liver infusion which is notoriously rich in these vitamins. They were needed, however, because addition of folic acid and riboflavin increased fertility and partly replaced, the liver infusion in medium F1 when increased above the levels in the vitamin mix. With the present media, no clear need was found for *p*-aminobenzoic, lipoic, and ascorbic acids, inositol and carnitine. Biotin, choline, cyanocobalamin, and putrescine were retained in the vitamin mixture, because they had shown some activity in several experiments, and for safety when

TABLE II

Dose response of water-soluble vitamins in F1 medium.

Vitamin (mg%)		Production in 7 days*	Vitamin (mg%)		Production in 7 days*
Thiamine HCl	0	8	Pyridoxine HCl	0	15
	0.4	86		0.2	90
	0.5	92		0.3	102
	0.6	98		0.4	104
	0.8	90		0.5	104
	1.0	80		0.6	76
Nicotinamide	0	21	Ca Pantothenate	0	34
	0.5	44		0.5	73
	1.0	58		2.0	100
	1.5	80		3.0	97
	2.0	95		4.0	107
	2.5	107		5.0	96
	3.0	88			

* Number of individuals produced by one female in 10 ml of medium at day 7.

replacing the liver infusion. Some vitamins had sharp optimal zones and inhibition at higher concentrations.

Nucleic acids were indispensable for fertility; their omission permitted the growth to adult of the original inoculum; but no progeny were produced, although the adult female remained alive up to three weeks. Adenylic acid is indispensable, not replaceable by guanylic acid, and seems to constitute the bulk of the nucleic-acid requirement. Best results were obtained with a mixture of adenylic, guanylic, cytidylic acids, and thymidine at a 2× concentration (Table III); lower or higher concentrations were less effective. Supplementation with various amounts of uridylic acid improved neither growth nor fertility. Adenine could not replace adenylic acid.

Cholesterol was indispensable and was supplied as a crystalline slurry (an ethanol solution injected in water) or as an emulsion with Tweens and bile acids (ethanol solution added to DF_2; see Table I). The optimal concentration was 0.6 mg%; 0.7 and 0.8 were still good but 0.9 mg and above was inhibitory; no attempts were made to replace it with the phytosterols used by insects.

A vitamin D requirement emerged in early experiments, when its incorporation into the egg-yolk particles permitted continuous generations in *Moina*. When omitted from the FV particles fertility of the F_2 was drastically lowered as shown by the small difference between the counts at day 7 and the counts after exhaustion

TABLE III

Effect of purine and pyrimidine nucleotides in F1 medium.

Nucleic acids (mg %)		Production in 7 days*
1. None		None
2. adenylic	40	15
3. adenylic	40	64
guanylic	20	
cytidylic	20	
4. adenylic	40	51
guanylic	20	
thymidine	20	
5. adenylic	40	42
cytidylic	20	
thymidine	20	
6. guanylic	20	14
cytidylic	20	
thymidine	20	
7. guanylic	30	23
cytidylic	30	
thymidine	30	
8. Nucleic mix V [adenylic 20, guanylic 10, cytidylic 10, thimidine 10]	1 ×	53
9. Nucleic mix V	2 ×	97
10. Nucleic mix V	3 ×	87
11. Nucleic mix V	4 ×	41

* Number of individuals produced by one female in 10 ml of medium at day 7.

TABLE IV

Production of Moina *in absence of fat-soluble vitamins and fatty acids in F1 medium.*

	Production in 7 days*	Final count (particles consumed)
Vitamin omitted		
vitamin A (or β-carotene)	69	159
vitamin D_2 (calciferol)	57	89
vitamin E (dl-α tocopherol)	41	79
None omitted	103	239
Fatty acids omitted		
palmitic acid	20	50
oleic acid	72	201
linoleic acid	87	168
linolenic acid	72	147
None omitted	98	251

* Number of individuals produced by one female in 10 ml of medium at day 7.

of the particles, *i.e.*, all particulate food was consumed but without increasing fertility (Table IV).

Omission of *dl*-α tocopherol acted similarly. Omission of β-carotene permitted lower but continued fertility, *i.e.*, higher counts at total consumption of particulate food (Table IV). To test whether vitamin E was an essential nutrient or its effect on growth and fertility was due to its antioxidant properties, the antioxidant BHT (butylated hydroxytoluene) was tried as a replacement for vitamin E (Table V). From the interchangeability of BHT and tocopherol, it was evident that the main action of tocopherol was that of antioxidant. Even though BHT at 2 mg% allowed excellent growth and fertility despite total absence of tocopherol,

TABLE V

Production of Moina *in F1 medium at various levels of antioxidants.*

(mg %) Vitamin E*/BHT	Production in 7 days**	Final count (particles consumed)
2.0/4.0	59	96
2.0/3.0	82	165
2.0/2.0	94	186
2.0/1.0	105	214
2.0/0.0	104	220
1.5/2.0	83	185
1.5/1.5	92	200
1.5/1.0	117	227
0.8/3.0	95	178
0.8/2.3	102	171
0.8/1.5	91	193
0/4.0	0	0
0/3.0	39	88
0/2.0	67	175

* 1 ml of tocopherol was assumed to equal 1 g.

** Number of individuals produced by one female in 10 ml of medium at day 7.

tocopherol alone or in combination elicited more growth and production of young, indicating that it might also be an indispensable nutrient.

Fatty acids were essential for growth and fertility as noted in the original substitution of serum and egg-yolk. Omission of palmitic acid curtailed fertility drastically; it was the only fatty acid which elicited linearly increased growth with increased concentration; production in 7 days at 0.5 mg% was 76 individuals; at 1 mg% 120 and 140 individuals at 1.5 mg%. Omission of linolenic seemed to affect fertility of the F_2 more than linoleic; oleic may be dispensable in the presence of other unsaturated fatty acids (Table IV).

Discussion

The main achievement of this study was the formulation of a nearly-defined medium which permits rapid growth and continued fertility of *Moina* for over 200 generations—a result reflecting recognition that fatty acids, phosphatides and vitamins A, E, D are essential for sustained fertility and that the way of presenting the needed nutrients is in itself crucial for rapid growth.

Work on *Artemia salina* (Provasoli and D'Agostino, 1969) had shown that particulate media solve the impasse of an inefficient uptake of solutes by supplying the bulk nutrients (protein, carbohydrate and lipids) in particulate form, thus complying with, and exploiting, the great ability of filter feeders to gather particles. The suspicion that lipids might be indispensable for fertility led to the use of coagulated egg-yolk and later to the formulation of a repeatable and more chemically defined particle which supplies the lipids in a readily acceptable form.

The present medium is defined except for liver infusion; replacement of this component, notoriously rich in growth factors, is in progress. Consequently, we could define only those needs which were demonstrable above the levels in liver infusion. Hence, while a clear need for thiamin, nicotinamide, pyridoxine and pantothenic acid could be demonstrated, the omission of folic acid and riboflavin reduced fertility noticeably, but not completely. A need for biotin could not be shown; it is needed by most insects, by *Artemia* and is probably needed by *Moina* also.

These seven vitamins are probably needed by other Cladocera; Murphy's success in rearing several species of Cladocera in monoxenic cultures with *Chlamydomonas reinhardii* depended upon increasing the concentrations of several B vitamins, among them choline and inositol. Under Murphy's conditions, addition of inositol in biweekly pulses, at a concentration which was inhibitory if added continuously, was essential to obtain several generations from newborn of the fourth and fifth orthoclone, increasing life-span and brood size (Murphy and Davidoff, 1972). Under our conditions we could not detect response to inositol or choline (which like inositol is needed by several insects). Since our media contain lecithin, either choline is not needed or the need is satisfied by 1 mg% lecithin.

Several B vitamins became inhibitory at supraoptimal concentrations, particularly folic acid and riboflavin. Excess vitamins were also inhibitory to several insects (Akov, 1962; Vanderzant, 1963; Horie, Watanabe and Ito, 1966), indicating that the widespread notion that overdoses of B vitamins are harmless is unjustified.

Nucleic acids are needed for fertility in *Moina*. The need for adenylic acid exceeds the requirement for other nucleotides. *Artemia* also requires nucleotides,

but they are already needed for growth from nauplii to adults; its requirement for adenylic acid is much higher than *Moina*, and *Artemia* also requires uridylic acid. While *Moina* and *Artemia* may be able to synthesize nucleotides, the concentrations required indicate that synthesis of nucleotides cannot keep pace with rapid growth.

Adenylic acid is apparently needed for ATP production, since Hernandorena (1974, 1975, 1976) found that the concentrations required by *Artemia* varied with concentrations of calorigenic nutrients (carbohydrates and fats) and that variations in temperature and salinity had opposite effects on the requirements for adenylic, protein and energetic nutrients. Most insects synthesize nucleic acids; but several Diptera, two beetles and the moth *Plodia* (Morère, 1974) need an exogenous source, perhaps because the rate of synthesis may be too low. In Diptera it has to sustain the thousand-fold increase in larval size which normally occurs in four days (House, 1972). In insects the need for nucleotides is satisfied by RNA and not by DNA; only *Agria affinis* utilizes both (House, 1964). While the house fly and *Drosophila* can utilize purine bases, adenine being the most important, *Agria affinis* like *Moina* and *Artemia* cannot utilize the purine and pyrimidine bases—it needs nucleotides.

A need for exogenous sterol is characteristic of insects and is met by cholesterol for all except *Drosophila palea*, which is monophagous on a cactus and needs schottenol or other $\Delta 7$ sterols. Stigmasterol, β-sitosterol and other phytosterols are equally well utilized by several phytophagous and omnivorous insects. A requirement for sterols may be common to all Arthropoda since ^{14}C-labeled precursors were not incorporated into sterols by the crab *Cancer* (van den Ord, 1964), the crayfish *Astacus* (Zandee, 1966), *Artemia* (Teshima and Kanazawa, 1971b), and prawn *Penaeus* and lobsters *Panulirus* (Teshima and Kanazawa, 1971c) and *Homarus* (Zandee, 1967), the spider *Avicularia* and the millipede *Graphidostreptus* (Zandee, 1967). *Artemia* and *Moina* need cholesterol, which seems to be better utilized than other sterols. Less effective utilization of phytosterols was also found for *Penaeus* (Kanazawa, Tanaka, Teshima and Kashiwada, 1971a). Teshima and Kanazawa (1971d) found that regardless of the sterol supplied (ergosterol, stigmasterol, beta-sitosterol and campesterol), *Artemia* contained only cholesterol; the prawn *Penaeus* behaved similarly (Kanazawa, Tanaka, Teshima and Kashiwada, 1971b). Cholesterol is apparently the only sterol found in several Crustacea (Teshima and Kanazawa, 1971a), indicating that the ability to convert dietary sterols into cholesterol may be wide-spread in Crustacea. Exogenous isotopically-labeled cholesterol contributed to the radioactivity of ecdysones in insects (Robbins, Kaplanis, Svoboda and Thompson, 1971) and of progesterone, androsterone and corticosterone in the hepatopancreas, ovaries and blood of the spiny lobster, *Panulirus* (Kanzawa and Teshima, 1971).

Vitamin D_2, calciferol, one of the isomers produced by UV-irradiation of ergosterol, is needed by *Moina* for fertility. *Moina* and many insects (Dadd, 1973) cannot use calciferol as a substitute for cholesterol. Calciferol, besides cholesterol, is needed to maintain fecundity, indicating that *Moina* presumably cannot synthesize it from cholesterol. A mixture (1 : 1) of ergosterol and olive oil absorbed on defatted yeast cells induced, in bacterized cultures of *Moina rectirostris*, production of 30% males and ephippial eggs (von Dehn, 1955).

Ergosterol is present in several species of phytoplankters and could be transformed into calciferol by irradiation, hence become available to Crustacea, even if it is not directly synthesized by the algae. The need for calciferol or its hydroxylated hormonal forms in insects might have been missed, because it was tested only as a substitute for cholesterol and because only recently has adequate attention been given to the requirements for continuous reproduction.

Fertility in *Moina* depends also on exogenous β-carotene or retinol and α-tocopherol. A vitamin E requirement for repairing ovarian disfunction and restoring fertility was first postulated by Viehoever and Cohen (1938), who suggested *Daphnia magna* for assay of the vitamin. The water-soluble sodium salt of dl-α-tocopherol phosphate lengthened the life of *Daphnia longispina* grown on yeast (Fluckinger and Fluck, 1950). Vitamin E was included early in insect diets to protect and delay oxidation of fatty acids, following the lead of Fraenkel and Blewett (1946) and Vanderzant, Kerur and Reiser (1957), but it was not recognized as a specific fertility factor until Chumakova (1962) reported that vitamin E was necessary for egg production of the beetle *Cryptolemus*. It is also necessary for viable sperms in the cricket *Acheta* (Meike and McFarlane, 1965), for a viable offspring in the parasitoid *Agria affinis* (House, 1966), for pupal development in the beetle *Oryzaephilus* (Davis, 1967), for the egg hatchability in the mite *Tetranychus* (Ekka, Rodriguez and Davis, 1971) and for female fecundity in the moth *Plodia* (Morère, 1971a). The concentration of tocopherol added to insect diets varied from 1 mg% to 200 mg% (for *Plodia*).

Apparently only Fraenkel and Blewett (1946) tried to discriminate between the antioxidant nonspecific effect and a specific effect on fertility. Though the antioxidant stabilization of fatty acids by tocopherol could be replaced by dietary ascorbic acid and ethyl or propyl gallate, they concluded that α-tocopherol has an independent growth promoting effect. This seems to apply to *Moina* for which good fertility for three generations was obtained with 2 mg% BHT in the absence of tocopherol, but addition of tocopherol to BHT resulted always in larger broods and better viability. Despite the pioneering work of Frankel, failure to detect the need for tocopherol in more insects is probably due to contamination of the lipids available in the early studies and to restriction of the nutritional analysis to one generation.

β-carotene and vitamin A are apparently essential for normal pigmentation in locusts (Dadd, 1961) and grasshoppers (Nayar, 1964) and synthesis of visual pigments, lack of which causes a loss of visual sensitivity (Goldsmith, Barker and Cohen, 1964; Zimmerman and Goldsmith, 1971). A direct effect on growth has been seldom observed; vitamin A improved larval growth in *Agria* (House, 1966) and carotene in locusts (Dadd, 1961) and *Plodia* (Morère, 1971b). Omission of carotene from the diet lowered the fertility of *Moina* noticeably but less than the omission of vitamin D and E and is apparently the first report for Crustacea. The claim of Dutrieu (1959) that carotenoids are essential for *Artemia* seems to be supported experimentally, if as yet weakly.

Moina requires saturated and unsaturated fatty acids. Omission experiments indicated that palmitic, linolenic, and linoleic promote fertility. Only palmitic acid was indispensable; increased fertility was a direct function of increased palmitate; omission of the other fatty acids lowered fertility, but less sharply. After dis-

covery that linoleic acid was needed for pupal eclosion and scale covering of wings in *Ephestia* (Fraenkel and Blewett, 1946), the dietary requirements for insects for saturated, mono- and poly-unsaturated fatty acids inspired a large literature (Dadd, 1973) and diverse requirements were found in different insect groups. Essentiality of polyunsaturated di- and trienoic C_{18} fatty acids is common in Lepidoptera, Orthoptera and Coleoptera; Diptera do not require them. Diptera may depend on a different lipid metabolism; ethanolamine phosphoglycerides and palmitoleic acid are their major lipids.

Isotopic studies indicate that insects synthesize saturated monoenoic fatty acids, and this seems to hold also for Crustacea (Zandee, 1967). Yet, especially in Diptera, palmitic and oleic acids are often needed for optimal growth. The importance of palmitic in *Moina* may be explained by the findings of Morris and Sargent (1973) that radioactive palmitic acid fed to, or injected in, Crustacea was used for biosynthesis of wax esters and high polyunsaturated fatty acids. Work with commercial diets for prawns and lobsters indicate that the ratio between ω3 and ω6 fatty acids may be an important factor.

It is evident then that Crustacea have requirements similar to insects, indicating that Arthropoda may have a rather homogeneous distinctive nutritional pattern. This similarity justifies, at least empirically in initial cultivation, the assumption that the general nutritional requirements of a group apply to a single species, but this cannot substitute for detailed studies.

Remarkable differences were found between *Artemia, Daphnia* and *Moina*. Even the initial attempts to grow *Daphnia* made it evident that the high starch/protein ratios (5 : 1) of *Artemia* did not suit *Daphnia* which preferred more protein. This was also true for *Moina*, for which the optimal range starch/protein lies between 1.5 : 1 and 0.5 : 1 ; within this range, the optimal ratio was influenced by the other components of the medium, particularly lipids. So far, egg albumin seems to satisfy the amino acid requirements of *Artemia* and *Moina*. For *Moina*, albumin fraction V inhibited above 8 mg% and could not replace egg albumin as the sole source of amino acids.

Nutrient ratios are well known to affect the efficiency of diets. Failure in attempts to shift the nutrition of invertebrates from natural food to artificial diet might be caused by improper ratios of nutrients, as well as by the selection of inadequate protein and lipid sources. Dietary efficiency is an important consideration for aqua-culture of shrimp, prawn and lobster. High protein diets seem to speed growth rates but also increase the cost of the diets, suggesting the need for more efficient carbohydrate : lipid : protein ratios. Extrapolation of our results indicated that the present diets may be deficient in nucleic acids and that means for preventing leaching of water soluble vitamins and minerals would greatly improve the efficiency of the commercial diets for animals which do not feed voraciously (Provasoli, 1976).

Summary

1. *Moina macrocopa* was cultured aseptically for more than 200 parthogenetic generations in a nearly-defined medium without losing fertility.

2. A biphasic medium was used. The liquid phase supplied minerals, B-vita-

mins, amino acids, liver infusion and nucleic acids. The fine particulate phase consisted of egg albumin, albumin fraction V, starch and lipid factors.

3. The particulate phase was essential for rapid growth, taking advantage of the food gathering efficiency of filter feeders.

4. Developmental time, brood size and sustained fertility depended on calciferol, tocopherol and saturated and unsaturated fatty acids; B carotene or retinol favored fertility but might not be essential.

5. *Moina* was found to require cholesterol, nucleic caids, thiamine, nicotinamide, pyridoxine, Ca pantothenate and probably riboflavin and folic acid in the presence of liver infusion, the only undefined and required component of the medium.

6. Ratios and quantities of the nutrients were important for media efficiency: excess vitamins could be inhibitory and the best starch/protein ratio was in the range of 1.5 : 1 to 0.5 : 1.

7. The requirements of *Moina* were compared with the range of requirements of arthropods and found to be similar to the nutritional patterns of phytophagous insects.

LITERATURE CITED

Akov, S., 1962. A qualitative and quantitative study of the nutritional requirements of *Aedes aegypti* larvae. *J. Insect Physiol.*, **8**: 319–335.

Chumakova, B. M., 1962. Significance of individual food components for the vital activity of mature predatory and parasitic insects. *Vop. Ekol. Kievsk.*, **8**: 133–134 (not seen in original, quoted from *Biol. Abstracts*, 45, 44, 502, 1964).

D'Agostino, A. S., and L. Provasoli, 1970. Dixenic culture of *Daphnia magna* Straus. *Biol. Bull.*, **139**: 485–494.

Dadd, R. H., 1961. Observations on the effect of carotene on the growth and pigmentation of locusts. *Bull. Entomol. Res.*, **52**: 63–81.

Dadd, R. H., 1973. Insect nutrition: current developments and metabolic implications. *Annu. Rev. Entomol.*, **18**: 381–420.

Davis, G. R. F., 1967. Effects of dietary lipids on survival and development of the sawtoothed grain beetle *Oryzaephilus surinamensis* (L.) (Coleoptera, Silvanidae). *Rev. Can. Biol.*, **26**: 119–124.

von Dehn, M., 1955. Die Geschlechtsbestimmung der Daphniden. Die Bedeutung der Fettstoffe untersucht an *Moina rectirostris*. *Zool. Jahrb. Abt. Allg. Zool. Physiol. Tiere*, **65**: 334–356.

Dutrieu, J., 1959. Les pigments *d'Artemia salina* L. Rapports entre la nature des pigments et le mode de reproduction des adultes. *C.R. Hebd. Seances Acad. Sci. Paris*, **248**: 2522–2524.

Ekka, I., J. G. Rodriguez, and D. L. Davis, 1971. Influence of dietary improvement on ovoposition and egg viability of the mite, *Tetranichus urticae*. *J. Insect Physiol.*, **17**: 1393–1399.

Fluckinger, E., and R. Fluck, 1950. Die Einfluss von Vitamin E auf *Daphnia longispina*. *Experientia*, **6**: 64.

Fraenkel, G., and M. Blewett, 1946. Linoleic acid, vitamin E and other fat soluble substances in the nutrition of certain insects (*Ephestia kuehniella, E. elutella, E. cautella* and *Plodia interpunctella* (Lep.). *J. Exp. Biol.*, **22**: 172–190.

Goldsmith, T. H., R. L. Barker, and C. F. Cohen, 1964. Sensitivity of visual receptors of carotenoid-depleted flies: a vitamin A deficiency in an invertebrate. *Science*, **146**: 65–67.

Hernandorena, A., 1974. Effects of salinity in the nutritional requirements of *Artemia salina*. *Biol. Bull.*, **146**: 236–248.

Hernandorena, A., 1975. Metabolic significance in nucleic acid metabolism and protein synthesis of dietary AMP requirement in *Artemia salina* L. *Biol. Bull.*, **148**: 416–428.

Hernandorena, A., 1976. Effects of temperature on the nutritional requirements of *Artemia salina*. *Biol. Bull.*, **151**: 314–321.

Horie, Y., K. Watanabe, and T. Ito, 1966. Nutrition of the silkworm, *Bombyx mori* XIV. Further studies on the requirements for B vitamins. *Bull. Seric. Exp. Stn. Tokyo.* **20**: 393–409.

House, H. L., 1964. Effects of dietetic nucleic acids and components on growth of *Agria affinis* (Fallen) (Diptera: Sarcophagidae). *Can. J. Zool.*, **42**: 801–806.

House, H. L., 1966. Effects of vitamin E and A on growth and development, and the necessity of vitamin E for reproduction in the parasitoid *Agria affinis* (Fallen) (Diptera: Sarcophagidae). *J. Insect Physiol.*, **12**: 409–417.

House, H. L., 1972. Insect nutrition. Pages 513–573 in R. N. Fiennes, Ed., *Biology of nutrition. Vol. 18.* Pergamon Press, Oxford.

Kanazawa, A., and S. Teshima, 1971. *In vivo* conversion of cholesterol to steroid hormones in the spiny lobster *Panulirus japonica*. *Bull. Jpn. Soc. Sci. Fish.*, **37**: 891–898.

Kanazawa, A., N. Tanaka, S. Teshima and K. Kashiwada. 1971a. Nutritional requirements of Prawn. II. Requirements for sterols. *Bull. Jpn. Soc. Sci. Fish.*, **37**: 211–215.

Kanazawa, A., N. Tanaka, S. Teshima, and K. Kashiwada, 1971b. Nutritional requirements of Prawn. III. Utilization of dietary sterols. *Bull. Jpn. Soc. Sci. Fish.*, **37**: 1015–1019.

Meike, J. E. S., and J. E. McFarlane, 1965. The role of lipid in the nutrition of the house cricket *Achcta domesticus* L. (*Orthoptera*, Grillidae). *Can. J. Zool.*, **43**: 87–98.

Morère, J. L., 1971a. Remplacement de l'huile de germe de mais par des acides gras dans l'alimentation de type meridique de *Plodia interpunctella* (Hbn.) (Lepidoptère-Pyralidae) *C.R. Hebd. Seances Acad. Sci. Paris*, **272**: 133–136.

Morère, J. L., 1971b. Le carotène: substance indispensable pour la nutrition de *Plodia interpunctella* (Lepidioptère-Pyralidae). *C.R. Hebd. Seances Acad. Sci. Paris*, **272**: 2229–2231.

Morère, J. L., 1974. Nutrition d'une chenille de lepidoptère *Plodia interpunctella* (Hbn) (Lepidoptère-Pyraldiae) sur un milieu chimiquement défini. *Arch. Int. Physiol. Biochim.*, **82**: 625–630.

Morris, R. J., and J. R. Sargent, 1973. Studies on the lipid metabolism of some oceanic Crustacea. *Mar. Biol.*, **22**: 77–83.

Murphy, J. S., 1970. A general method for the monaxenic cultivation of the Daphnidae. *Biol. Bull.*, **139**: 321–332.

Murphy, J. S., and M. Davidoff, 1972. The result of improved nutrition on the Lansing effect in *Moina macrocopa*. *Biol. Bull.*, **142**: 302–309.

Nayar, J. K., 1964. The nutritional requirements of grasshoppers. I. Rearing of the grasshopper *Melanoplus bivattatus* (Say) on a completely defined diet and some effects of different concentrations of B vitamin mixtures, linolenic acid and β-carotene. *Can. J. Zool.*, **42**: 11–22.

Provasoli, L., 1976. Nutritional aspects of crustacean aquaculture. Pages 13–21 in K. S. Price, M. N. Shaw, and K. S. Danberg, Eds., *Proc. First Int. Conf. Aquaculture Nutrition*, University of Delaware, Newark, Delaware.

Provasoli, L., and A. S. D'Agostino, 1969. Development of artificial media for *Artemia salina*. *Biol. Bull.*, **136**: 434–453.

Robbins, W. E., J. M. Kaplanis, J. A. Svoboda, and M. J. Thompson, 1971. Steroid metabolism in insects. *Annu. Rev. Entomol.*, **16**: 53–72.

Teshima, S., and A. Kanazawa, 1971a. Sterol compositions of marine crustaceans. *Bull. Jpn. Soc. Sci. Fish.*, **37**: 63–67.

Teshima, S., and A. Kanazawa, 1971b. Utilization and biosynthesis of sterols in *Artemia salina*. *Bull. Jpn. Soc. Sci. Fish.*, **37**: 720–723.

Teshima, S., and A. Kanazawa, 1971c. Biosynthesis of sterols in the lobster *Panulirus japonica*, the prawn *Penaeus japonicus*, and the crab *Portunus trituberculatus*. *Comp. Biochem. Physiol.*, **38B**: 597–602.

Teshima, S., and A. Kanazawa, 1971d. Bioconversion of the dietary ergosterol to cholesterol in *Artemia salina*. *Comp. Biochem. Physiol.*, **38B**: 603–607.

Van den Ord, A., 1964. The absence of cholesterol synthesis in the crab *Cancer pagurus*. *Comp. Biochem. Physiol.*, **17**: 715–718.

VANDERZANT, E. S., 1963. Nutrition of the boll weevil larva. *J. Econ. Entomol.*, **56**: 357–362.

VANDERZANT, E. S., D. KERUR, AND R. REISER, 1957. The role of dietary fatty acids in the development of the pink bollworm. *J. Econ. Entomol.*, **50**: 606–608.

VIEHOEVER, A., AND J. COHEN, 1938. The response of *Daphnia magna* to vitamin E. *Am. J. Pharm. Educ.*, 110: 297–315.

WICKSTEAD, J. H., 1965. *An introduction to the study of trophical plankton.* Hutchinson and Co., London, 160 pp.

ZANDEE, D. I., 1966. Metabolism in the crayfish *Astacus astacus*. III. Absence of cholesterol synthesis. *Arch. Int. Physiol. Biochim.*, **74**: 435–441.

ZANDEE, K. I., 1967. Absence of cholesterol synthesis as contrasted with the presence of fatty acids synthesis in some arthropods. *Comp. Biochem. Physiol.*, **20**: 811–822.

ZIMMERMAN, W. F., AND T. H. GOLDSMITH, 1971. Photosensitivity of circadian rhythms and of visual receptors in carotinoid-depleted *Drosophila*. *Science*, **171**: 1167–1169.